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14. ABSTRACT

TGFβ provided by the bone microenvironment is a key factor in the development of bone metastases. Previous experiments have demonstrated that interference with TGFβ signaling in cancer cells decreases the development of bone metastases. TGFβ stimulates prostate cancer cell signaling and alters their phenotype. TGFβ signaling in cancer is however complex and can lead to the activation of numerous genes. We identified PMEPA1 as the most highly unregulated gene by TGFβ in PC3 cells. Although PMEPA1 has already been shown to be unregulated in different cancers, nothing is known about its function in cells. We have shown that the absence of PMEPA1 in prostate cancer cells decreases TGFβ signaling. This result is consistent with preliminary experiments showing that the cytosolic isoform of PMEPA1 which is the most highly expressed in PC3 increases TGFβ signaling. Interestingly the other isoforms of PMEPA1 which are membrane bound have an opposite effect, decreasing TGFβ signaling. These results suggest that depending on which isoform is the most abundant in cells, PMEPA1 can provide a positive or negative feedback loop for TGFβ signaling. We are in the process of stably knocking down and over expressing PMEPA1 in PC3 cells to determine the effect of PMEPA1 on bone metastases development.

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INTRODUCTION

Metastasis, the ultimate step of malignancy, is not a randomized process and some sites, such as bone, are preferential targets [1]. At least 65% of patients with advanced breast or prostate cancer will develop bone metastases [2]. Tumor cells in bone disrupt normal bone remodeling to cause an excess in bone destruction and/or bone formation which fuel a vicious cycle [3]. Bone metastases are associated with severe consequences: pain, hypercalcemia, fractures, or nerve compression syndromes and paralysis that drastically reduce quality of life. Moreover, once metastatic cells colonized bone, there is no cure, only palliation. Bone metastases are a major public health problem. Thus, a detailed understanding of the bone metastases process is needed to define targets and design new treatments to cure cancer.

Several studies showed that transforming growth factor-β (TGF-β) is a major mediator of metastasis: it activates epithelial-mesenchymal transition (EMT) [4] and tumor cell invasion [5], increases angiogenesis [6] and induces immunosuppression [7]. TGF-β is also crucial in the bone metastases process [8-10]. Bone is a major source of TGF-β since it is synthesized and trapped in the mineralized matrix by the osteoblasts. It is released and activated during osteoclastic bone resorption [11]. TGF-β acts then on cancer cells to induce the secretion of prometastatic factors such as interleukin (IL)-11 [12], endothelin-1 (ET-1) [13] or parathyroid hormone-related protein (PTHrP) [10]. These proteins in turn induce either an osteoblastic response (ET-1) or osteolysis (PTHrP) [14; 15].

We recently identified a new downstream target of TGF-β in the prostate cancer cells PC-3, using micro-array technology. This gene, PMEPA1, was the most highly upregulated by TGF-β (23-fold increase, $P<0.03$). It codes for proteins overexpressed in breast, ovarian and colorectal cancers [16; 17]. Although the function of PMEPA1 is unknown, it has been shown to interacts with Nedd4 [18; 19], a member of the Nedd4 family of HECT domain E3 ubiquitin ligases (which also includes Smurf1 2, and Tiul1) [20]. HECT E3 ubiquitine ligases are implicated in the degradation of Smad proteins and the regulation of the TGF-β signaling pathway [21; 22].

Bone is the most common site for prostate and breast cancer metastasis. Bone-derived TGF-β contributes to this process in many ways: (1) as a prometastatic factor (i.e., EMT, invasion, angiogenesis) and (2) as an inducer of bone active factors (i.e., PTHrP, IL-11 and ET-1) (**Figure 1**). We propose here a new mechanism by which TGF-β promotes bone metastases with the induction of PMEPA1 at the bone metastatic site. *In this proposal, we will test the **hypothesis** that TGF-β released from bone at the site of bone metastases induces the production of PMEPA1. PMEPA1 expression is important for bone metastases development, by interacting with HECT E3 ubiquitin ligases to prevent the degradation of Smad proteins. This results in a continuous activation of the TGF-β pathway in cancer cells housed in bone and perpetuates the vicious cycle to result in the production of more osteolytic and osteoblastic factors.*

Three specific aims will be addressed. **Aim 1**: to characterize the TGF-β induced transcription of PMEPA1 *in vitro*. **Aim 2**: to assess the TGF-β induced upregulation of PMEPA1 and its role in bone metastases formation *in vivo*. **Aim 3**: to characterize the interaction of PMEPA1 with E3 ubiquitin ligases and its involvement in TGF-β signaling regulation.

BODY

Specific Aim 1: To determine the TGF-β induced transcription of PMEPA1 in vitro

Task 1: Analysis of PMEPA1 variants expression after TGF-β treatments in vitro

The PMEPA1 gene covers 63kb. Alternative splicing and multiple transcription starts give rise to 4 different mRNA variants (Genbank accession numbers: NM_020182, NM_199169, NM_199170 and NM_199171 for variants 1, 2, 3 and 4 respectively). These mRNA are highly similar except in the 5' extremity and code for 3 different protein isoforms that differ at their N-terminus. Isoform a and b both contain a transmembrane domain, while isoform c, the shortest, is cytosolic.

To determine which mRNA variant is the most abundant in PC-3 cells treated or not treated with TGF-β, we used absolute quantitative real-time RT-PCR.

Primers specific for each variants of PMEPA1 have been designed and their amplification product was cloned in a pSC-A linearized according to the manufacturer's instruction (StrataClone™ PCR Cloning Kit, Stratagene) and used to establish standard curves for real-time PCR. Similarly we cloned the amplification products of the primers for the endogenous ribosomal protein L32 to normalize our RT-PCR. PC-3 cells were grown until they reach near confluency and starved in basal media overnight before being treated or not-treated with TGF-β (5ng/mL) for 24 hours. Total RNA was extracted from cell (RNeasy mini-kit, Qiagen) and treated with DNase I to avoid DNA contamination (RNase free DNase set, Qiagen). cDNA were obtained using the SuperScript ™ II reverse transciptase (Invitrogen) and used as template in an absolute quantitative real-time PCR (QuantiTect SYBR green PCR kit, Qiagen) using a BioRad MyiQ thermocycler.

TGF-β significantly increased the expression of all PMEPA1 messenger RNA variants, and variant 2 has the highest induction (≈20-fold) (**Figure 2**). Variant 1 coding for isoform a of the protein has the lowest expression in the presence or absence of TGF-β. Variant 3 and 4 code for the same protein, isoform c but remain less abundant then variant 2 which the most abundant mRNA, in the presence of TGF-β, suggesting that PMEPA1 isoform b is the most abundant in TGF-β-stimulated PC-3 cells. However when we quantified the number of copy of PMEPA1 mRNA using a pair of primer designed in the 3' extremity of the mRNA, we calculated a total amount of mRNA ≈11-times superior to the addition of all the mRNA variants we measured (**Figure 2**). Due to the low nucleotide variation between each variant, we could not change their specific primers to determine whether it would affect the quantification, so we designed a new pair of primers targeting a region closer to the 5' extremity identical to all variants. However both pair of primers for total PMEPA1 detected similar quantities of mRNA (data not shown).

Since we could not solve the problems linked to mRNA quantification, we used an alternative approach with a new antibody against PMEPA1. This antibody was not available when we initiated the project and wrote the proposal which was considered as a weakness by the Reviewer A during the reviewing process. Before trying to develop our own antibody, we kept on searching manufacturer's catalogs and found a mouse monoclonal antibody directed against an epitope in the C-terminus of PMEPA1 protein (Abnova) which had been recently released. The C-terminus of PMEPA1 is identical to all isoforms and this antibody should detect all of them. To test the antibody, the coding sequence of each PMEPA1 isoform was amplified by PCR using a *Pfu* proof-reading DNA polymerase (Stratagene) and the IMAGE clone 4559576 as a template and the following oligonucleotide primers: forward primer for isoform a 5'-CTAGCTAGCTAGACCATGCACCGCTTG ATGGGGGTCAACAGCACCGCCGCCGCCG-3', forward primer for isoform b 5'-CTAGCTAGCTAGACC ATGGCGGAGCTGGAGTTTGTTCAG-3', forward primer for isoform c 5'- CTAGCTAGCTAGACCATGATGG TGATGGTGGTGGT-3', and the reverse primer 5'-CCCAAGCTTGGG GAGAGGGTGTCCTTTCTGTT-3' for all the isoforms. The amplification products were cloned in a pcDNA3.1-Zeo+ plasmid between the NheI and HindIII restriction sites. We also inserted at the 3' end a V5 epitope tag and a stop codon between the KpnI and EcoRI restriction sites of the plasmid using the following oligonucleotide 5'-CGGGGTACCCCGGGTAAG CCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGTAGCCG GAATTCCGG-3' and its complementary strand. pcDNA3.1 plasmids expressing LacZ or the different isoforms of PMEPA1-V5 protein were

transfected in COS-1 cells. Protein lysates were prepared 24 hours later and analyzed by Western-blotting to detect first PMEPA1 and then V5 (anti-V5 antibody, Sigma) after stripping the membrane. Immunodetection of α-tubuline (anti-α-tubuline antibody, Sigma) was used to confirm the loading of equal amount of proteins.

A signal was detected at the expected molecular weights with the antibody against PMEPA1 only in the wells containing PMEPA1-V5 proteins, not LacZ (**Figure 3**). This signal was similar to the one obtained with the anti-V5 antibody (**Figure 3**). Multiple bands are detected in the protein lysate of the COS cells transfected to express PMEPA1a-V5, by the antibodies against PMEPA1 and V5. It suggests that these bands are due to multiple proteins expressed by the plasmid transfected, while restriction maps and sequencing confirmed the purity and the identity of the plasmid. This result has not been explained yet but does not prevent the use of this new antibody to detect PMEPA1 using Western-Blot.

To characterize the effect of TGF-β on PMEPA1 endogenous protein expression, PC-3 cells were treated with increasing concentration of TGF-β (from 0.1 to 5ng/mL) for 24 hours. Using western-blotting, PMEPA1 was not detected in untreated PC-3 cells (**Figure 4A**). A concentration of TGF-β as little as 0.1ng/mL allowed the detection of a single band with an apparent molecular weight of ≈35kDa and TGF-β-induction of this band appeared maximal at 0.25ng/mL (**Figure 4A**). Additional bands of lower intensity, corresponding to protein of higher molecular weight were detected when PC-3 cells were treated with 0.25 to 5.0ng/mL of TGF-β (Figure 4A). Protein lysates of COS cells transfected to express one of the PMEPA1 isoform were run next to the PC-3 endogenous proteins and showed that the band of highest intensity detected in PC-3 lysates corresponds to the isoform c of PMEPA1 which lacks the transmembrane domain (**Figure 4A**).

When PC-3 cells were treated or not treated with 5ng/mL of TGF-β up to 48 hours before preparing protein lysates. Immunodetection showed that PMEPA1 protein was detected after 4 hours of TGF-β treatment and increased until 24 hours when it reached a plateau (**Figure 4B**). Expression of PMEPA1 induced by TGF-β in PC-3 cells was prevented by using SD-208, a small molecule inhibitor of the kinase activity of the TGF-β type I receptor (Tgfbr1) (**Figure 4C**).

Conclusion: We demonstrated that PMEPA1 protein expression is quickly and stably induced by TGF-β in PC-3 human prostate cancer cells. Expression of PMEPA1 is also very sensitive to TGF-β. Although the expression of all mRNA variants was detected and increased by TGF-β in PC-3 cells, it is the isoform c of PMEPA1 which is the most abundant. We are planning to test the expression of PMEPA1 protein in other prostate cancer cells (DU145, LuCap23.1, LnCap, C4-2B) but also breast (MDA-MB-231) and lung (A549) cancer, and hepatocarcinoma (HepG2) cells.

Task 2: Characterization of TGF-β induction of PMEPA1 and of PMEPA1 promoter

To further understand the mechanism of TGF-β-induced expression of PMEPA1, PC-3 cells were treated up to 48 hours with TGF-β (5ng/mL) in the presence or absence of the specific Tgfbr1 inhibitor, SD-208. Total mRNA were prepared and the expression of all PMEPA1 mRNA variants was measured by real-time semi-quantitative RT-PCR. PMEPA1 mRNA is quickly increased by TGF-β and reaches a pick after 4 hours (**Figure 5**). PMEPA1 upregulation by TGF-β was totally inhibited by SD-208 (**Figure 5**). We also used the classical cycloheximide and actynomycin-D, at concentrations inhibiting PC-3 cell growth. The translation inhibitor cycloheximide did not reverse TGF-β effect showing that PMEPA1 is a direct target gene of the TGF-β pathway (**Figure 5**). The transcription inhibitor, actinomycin D prevented the increase of PMEPA1 mRNA suggesting that TGF-β does not increase the mRNA stability (**Figure 5**).

These results show that TGF-β regulates PMEPA1 expression through transcriptional control.

The analysis of PMEPA1 promoter (up to 3.7 kb upstream of the transcription start of mRNA variant 1 of PMEPA1) showed the presence of 5 consensus Smad binding elements (SBE), 5'-CAGACA-3' (**Figure 6**). This fragment of the PMEPA1 promoter was cloned from a cosmid (BACPAC resources, CHORI) into the pGL3 plasmid, a *firefly* luciferase reporter vector (Promega). The pGL3-PMEPA1 plasmid was co-transfected with the ph*r*Luc-CMV plasmid (Promega), which constitutively expresses *renilla* luciferase, in

PC-3 (prostate cancer cells), and HepG2 (hepatocarcinoma) and A459 (lung cancer cells) known for their sensitivity to TGF-β. These cells were treated or not treated with TGF-β (5ng/mL, 24 hours) before measuring dual-luciferase activity with a FB15 Sirius luminometer (Zylux corporation). TGF-β significantly increased PMEPA1 promoter activity in the 3 cell lines tested which was reversed by the Tgfbr1 inhibitor, SD-208 (**Figure 7**). Moreover when A549 cells were transfected to overexpress Smad2, 3 and 4, the TGF-β pathway effectors, PMEPA1 promoter was increased independently of TGF-β (**Figure 7**). Conversely TGF-β-induced activity of PMEPA1 promoter was significantly inhibited by the overexpression of the Smad inhibitor, Smad7 (**Figure 7**). These results concur to show that TGF-β-induction of PMEPA1 involves Smad proteins.

Conclusion: TGF-β regulates the expression of PMEPA1 through transcriptional control via a molecular mechanism involving Smad proteins.

Task 3: Identify the SBEs and DNA motifs involved in TGF-β regulation of PMEPA1 promoter

Therefore we performed site-directed mutation of the SBEs using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. The five 5'-CAGACA-3' SBEs identified in the 3.7kb fragment were respectively mutated to 5'-tAcAtA-3', 5'-CctgCA-3', 5'-tAGAtA-3', 5'-CAtgtA-3' and 5'-tActCA-3', each creating a new restriction site, which was used to confirm the introduction of each mutation. These constructs with phRL-CMV were transfected in A549 cells and the cells were treated or not treated with TGF-β (24h, 5ng/mL) before measuring the dual-luciferase activity. Mutation of one or all of the SBE did not significantly decrease the promoter activity induced by TGF-β as we would have expected (**Figure 8A**). This lack of effect could be due to additional DNA motifs in the promoter binding Smad proteins or non-Smad protein activated by the non-Smad TGF-β signaling. Thus we tested the effect of the overexpression of the effectors Smad2/3/4 or the inhibitor Smad7 on the activity of the PMEPA1 promoter where the 5 SBEs have been mutated. Mutation of the 5 SBEs decreased the promoter activity induced by Smad2/3/4 when compared to the wild-type promoter, which confirm that these elements are involved but not necessary to the TGF-β induction (**Figure 8B**). However overexpression of Smad7 still decreased the promoter activity induced by TGF-β in presence of the mutated SBEs (**Figure 8B**), demonstrating that TGF-β induction of PMEPA1 promoter is independent of the predicted SBEs.

To determine which region of the promoter is responsible for the TGF-β inducibility, we tested the TGF-β responsiveness of a series of promoter deletion constructs in A549 cells using dual-luciferase assay. Deletion of the first 2.5kb of the promoter, nucleotides -3691 to -1440, did not significantly affect the increased promoter activity induced by TGF-β when compared to the 3.7kb promoter (**Figure 9**). In the remaining 1.5kb fragment, deletion of the first 900bp or last 600bp resulted in a 50% decrease of the PMEPA1 promoter activity induced by TGF-β (constructs -530/+54 and -3691/-484, respectively), and the full activity of the promoter was not restored when the distal 2.5kb of the promoter were added (construct -3691/-484, **Figure 9**). Of note, a distal fragment of the promoter containing 4 of the 5 SBE cloned in front a minimal promoter was not activated when the cells were treated with TGF-β (construct -3691/-1856, **Figure 9**). These results suggest that the TGF-β responsive elements in the PMEPA1 promoter are located within the nucleotides -1440 and +54.

All the fragments of the PMEPA1 promoter containing SBE, showed an increase of luciferase activity in the presence of TGF-β as well as the -530/+54 fragment, which does not contain any SBE, 5'-CAGACA-3' (**Figure 9**). So we co-transfected the A549 cells with Smad2, 3 and 4 expression vectors and with the promoter fragments carrying SBE. Smad protein overexpression increased the basal promoter activity, however in the -530/+54 proximal fragment without SBE, Smad protein overexpression did not increase the basal promoter activity suggesting that the promoter activity induced by TGF-β is mediated by non-Smad mechanisms (**Figure 9**). To further analyze this 1.2kb fragment of the PMEPA1 promoter, we tested the effect of Smad2/3/4. Overexpression of the Smad effectors significantly increased the promoter activity of the full length of the PMEPA1 promoter and of the 1.2kb fragment in absence of TGF-β (**Figure 10**). The basal promoter activity of the proximal 600bp (construct -530/+54) was not affected by Smad2/3/4 but remained increased when cells were treated with TGF-β (**Figure 10**), suggesting that TGF-β responsiveness of this fragment is mediated through non-Smad mechanisms.

Conclusion: These results show that TGF-β regulates PMEPA1 expression through transcriptional control. PMEPA1 expression induced by TGF-β is independent of the predicted SBEs. TGF-β induction of the PMEPA1 promoter is controlled by a 1.2kb proximal fragment via both Smad and non-Smad mechanisms. As of now, the elements responsible for TGF-β induction of the PMEPA1 promoter remain to be identified.

Further analysis of the 1.2kb sequence and use of DNAse 1 footprinting could be used to identify the elements responsible for TGF-β induction. To confirm that TGF-β controls PMEPA1 expression through Smad and non-Smad mechanisms, we will test the effect of inhibitors directed against p38, MEK1/2 or JNK, kinases involved in non-Smad TGF-β signaling, in dual-luciferase experiment with the full length promoter or the 1.2kb fragment, and on PMEPA1 mRNA expression in PC-3 cells.

Specific Aim 2: Assess the TGF-β-induced expression of PMEPA1 in vivo

Task 4: In vivo quantification of PMEPA1 expression at sites of bone metastases in a prostate cancer bone metastases model

We have chosen to use species specific sqRT-PCR to measure PMEPA1 expression in bone marrow samples of mice with PC-3 bone metastases treated or not treated with SD-208, an inhibitor of the TGF-β type I receptor (tgfbr1).

The primers used to measure human PMEPA1 and RPL32 (housekeeping gene) mRNA by sqRT-PCR were designed to be specific of the human mRNA and ignore mouse mRNA. RT-PCR with RNA from PC-3 human prostate cancer cells or from mouse bone marrow cells were performed and amplification product were run on agarose gel to control that the primers do not amplify any product from mouse templates (data not shown). We also validated that the RT-PCR efficiency for the human PMEPA1 and RPL32 remained unchanged in the presence or absence of mouse template. The efficiency of the PCR was measured by use of a dilution series of PC-3 cDNA in water or in mouse cDNA to generate amplification curves at different concentration of template. Primers for human RPL32 and human PMEPA1 with efficiency of 100 ± 5% were selected (data not shown).

SD-208 is a pteridine derivative that specifically inhibit the serine kinase activity of the tgfbr1 (IC_{50} = 70nM). We confirmed that SD-208 decreased TGF-β signaling in PC-3 cells using western blotting to detect the phosphorylation of Smad2 and dual-luciferase experiment (**Figure 11**).

To induce osteolytic bone metastases, athymic male mice were inoculated in the left cardiac ventricle wit PC-3 prostate cancer cells. Osteolysis development was surveilled by serial radiography and mice (n=14/group) received SD-208 (50mg/kg/day, po) or the vehicle throughout the whole protocol. SD-208 significantly decreased the osteolysis area induced by PC-3 cells (vehicle 15.3 ± 2.8mm², SD-208 6.7 ± 3.3mm², 56% inhibition, P<0.05) (**Figure 12**). However SD-208 did not have any effect on the human PMEPA1 mRNA expression measured by sqRT-PCR in the bone marrow of metastastic tibia and femur collected 54 days after tumor inoculation (**Figure 13**). As a control we measured the expression of the human PTHrP since it has been well established that bone-derived TGF-β induces PTHrP expression at sites of bone metastases, and similarly to PMEPA1 PTHrP expression was not affect by SD-208 (**Figure 13**). Although SD-208 significantly decreased tumor-induced osteolysis, there is still bone destruction and therefore release of TGF-β from the bone matrix. Considering that concentrations of TGF-β has low as 250pg/ml induced PMEPA1 expression *in vitro* (**Figure 4**), it is likely that local concentrations of TGF-β at sites of bone metastases treated with SD-208 were high enough to induce PMEPA1 expression. It is also possible that other factors present at sites of bone metastases induced PMEPA1 expression independently of TGF-β.

As a collaboration with Dr. H. Frierson from the Department of Pathology at the University of Virginia, we validated the antibody against PMEPA1 for immunohistochemistry. We are planning to perform immunostaining of tissue sections of mouse metastatic bones to confirm whether PMEPA1 protein is expressed in PC-3 cells in bones when mice were treated or not treated with SD-208.

Task 5: Effect of PMEPA1 knockdown on the development of bone metastases from prostate cancer cells in mice

To knockdown PMEPA1 expression in PC-3 cells, we used pLKO.1 vector expressing predesigned

shRNA (Sigma) and selected one which efficiently targets PMEPA1. COS7 monkey kidney cells were transfected with a vector expressing one of the PMEPA1 isoform and an empty pLKO.1 vector or a pLKO.1 vector expressing a shRNA control (shControl) or directed against PMEPA1 (shPMEPA1). PMEPA1 expression was assessed 48 hours later. Using real-time RT-PCR, we measured a 90% decrease of expression of any PMEPA1 mRNA variant induced by shPMEPA1 (**Figure 14A**). The empty pLKO.1 vector or a vector expressing shControl had no effect on PMEPA1 expression. By western blot, we confirmed that shPMEPA1 prevented PMEPA1 expression regardless of the isoform (**Figure 14B**).

PC-3 cells were then transfected with a pLKO.1 vector expressing shControl or shPMEPA1. Selection of the clones was obtained after growing the cells for 2 weeks in the presence of puromycin (250ng/ml) and using cloning cylinders. Selection of clones was based on the absence of PMEPA1 protein expression when the cells were treated with TGF-β, using western-blotting. Eight clones where PMEPA1 expression was knocked-down were selected for further analysis and are currently being tested for stability of the knock-down during 70 days in the absence of puromycin (data not shown). Two of these clones will be selected to be inoculated in the left cardiac ventricle of athymic mice to cause bone metastases compared to clones expressing shControl or untransfected cells during year 2.

Task 6: Effect of PMEPA1 expression overexpression on the development of bone metastases from prostate cancer in mice.

PC-3 cells where the endogenous expression of PMEPA1 was knocked-down using shPMEPA1 (see Task 5) will be stably transfected to specifically express one of PMEPA1 protein isoform. For that purpose, 4 silent mutations were inserted in the nucleotide sequence recognized by the shPMEPA1 localized in PMEPA1 CDS, coded by a pcDNA3.1 vectors. Mutation of 4 nucleotides was sufficient to decrease the efficiency of shPMEPA1 and allow the re-expression of PMEPA1 protein (**Figure 15**).

Stable transfection of PC-3 shPMEPA1 clones will be performed during year 2 to test the effect of PMEPA1 overexpression on bone metastases development during year 2.

Specific Aim 3: Characterize the interaction of PMEPA1 with Smurf proteins and its involvement in TGF-β signaling regulation

Task 7: Characterize the interaction of PMEPA1 with Smurf proteins

We validated the newly available mouse monoclonal antibody against human PMEPA1 for immunoprecipitation experiments (**Figure 16**). This antibody will be used to determine the interaction of PMEPA1 with Smurf proteins during year 2.

Task 8: Determine the effect of PMEPA1 overexpression or knockdown on TGF-β signaling.

To assess TGF-β signaling activity in PC-3 prostate cancer cells, we used a pGL3 reporter vector where luciferase expression is controlled by a 9 CAGA boxes (Smad binding motifs) in dual-luciferase experiments. Furthermore, PC-3 cells were co-transfected with an expression plasmid coding for the isoform c of PMEPA1, the most abundant isoform in PC-3, or LacZ as a control, and the cells were treated or not treated with TGF-β before measuring luciferase activity. In a preliminary experiment, the isoform c of PMEPA1, the cytosolic isoform, induced a non significant increase of TGF-β signaling as shown by the increase of the $(CAGA)_9$ promoter activity (**Figure 17B**). The non-significant effect of PMEPA1c overexpression coud be due to the presence of the endogenous protein in PC-3 cells. Therefore we used the opposite strategy where PMEPA1 expression was knockdown in PC-3 cells using a vector expressing the validated shRNA against PMEPA1. PMEPA1 knockdown significantly decreased $(CAGA)_9$ promoter activity in PC-3 cells suggesting that PMEPA1 increases TGF-β signaling (**Figure 17A**).

We also tested the effect of the overexpression of PMEPA1 a and b, the membrane-bound isoform of PMEPA1, on TGF-β signaling. As shown on **Figure 17B**, PMEPA1 a and b induced a significant decrease of the $(CAGA)_9$ promoter activity. These results suggest that depending on its cellular localization PMEPA1 protein can be either an inducer or an inhibitor of TGF-β signaling in PC-3 prostate cancer cells.

Additional experiments using PMEPA1 proteins where the PPxY domain has been mutated are being performed to characterize the involvement of the E3 ubiquitin ligases Smurf in PMEPA1 function during year 2.

Task 9: Determine the effect of PMEPA1 overexpression or knockdown on the activity of Smad proteins.

Will be addressed during year 2.

KEY RESEARCH ACCOMPLISHMENTS

1. The expression of the gene PMEPA1 is regulated by TGF-β in a dose- and time-dependent manner.
2. TGF-β regulates PMEPA1 expression through transcriptional control via Smad and non-Smad mechanisms. TGF-β regulates PMEPA1 promoter activity independently of the predicted Smad Binding Elements.
3. TGF-β increases PMEPA1 expression in different TGF-β sensitive prostate, breast and lung cancer cell lines.
4. The cytosolic isoform PMEPA1c is the most abundant isoform of PMEPA1 induced by TGF-β in PC-3 prostate cancer cells.
5. Knockdown of PMEPA1 expression in PC-3 prostate cancer cells decreases TGF-β signaling.
6. The cytosolic isoform of PMEPA1 appears to increase TGF-β signaling in prostate cancer cells.
7. The membrane-bound isoforms of PMEPA1 decrease TGF-β signaling in prostate cancer cells.

REPORTABLE OUTCOMES

Publications

1. D. Javelaud, K.S. Mohammad, C.R. McKenna, **P. Fournier**, F. Luciani, M. Niewolna, J. Andre, V. Delmas, L. Larue, T.A. Guise, and A. Mauviel. *Stable overexpression of Smad7 in human melanoma cells impairs bone metastasis*. Cancer Research. 67(5):2317-2324. 2007.
2. **PG. Fournier**, and TA. Guise. *BMP7: a new Bone Metastases Prevention?* American Journal of Pathology. 171(3):739-43. 2007.
3. LA. Kingsley, **PG. Fournier**, JM. Chirgwin, and TA. Guise. *Molecular biology of bone metastasis*. Molecular Cancer Therapeutics. 6(10):2609-17. 2007.

Oral Presentations

1. **PG. Fournier**, GA. Clines, JM. Chirgwin, and TA. Guise. *TGF-β Promotes Prostate Cancer Bone Metastases and Increases the Expression of Pro-Osteolytic Genes and of the TGF-β Signaling Regulator PMEPA1*. Skeletal Complications of Malignancy V. Paget Foundation. Philadelphia, PA, USA. 2007.
2. **PG. Fournier**, GA. Clines, JM. Chirgwin, and TA. Guise. *TGF-β Promotes Prostate Cancer Bone Metastases and Increases the Expression of Pro-Osteolytic Genes and of the TGF-β Signaling Regulator PMEPA1*. The 25th Annual American Cancer Society Virginia Cancer Researchers Seminar, Richmond, VA, USA. 2007.
3. **PG. Fournier**. *Mechanisms and treatment of bone metastases - Role of TGF-β and use of bisphosphonates*. Invitation from A. Mauviel, INSERM U697, Hôpital Saint Louis, Paris, France. 2007.
4. **PG. Fournier**. *TGF-β Signaling and Bisphosphonates in Bone Metastases*. Invitation from G. van der Pluijm, Leiden University Medical Center, Netherlands. 2007.

Posters

1. **PG. Fournier**, GA. Clines, JM. Chirgwin, and TA. Guise. *TGF-β Promotes Prostate Cancer Bone Metastases and Increases Expression of Pro-Osteolytic Genes and of the TGF-β Signaling Regulator PMEPA1*. FASEB Summer Research Conferences. TGF-β Superfamily: Signaling and Development. Tucson, AZ, USA. 2007.
2. **PG. Fournier**, GA. Clines, JM. Chirgwin, and TA. Guise. *TGF-β Increases Osteolytic Prostate Cancer Bone Metastases and Expression of Pro-Metastatic Genes.* American Society for Bone and Mineral Research 29th annual meeting. Honolulu, HI, USA. 2007.

CONCLUSIONS

TGF-β provided by the bone microenvironment is a key factor in the development of bone metastases. Previous experiments have demonstrated that interference with TGF-β signaling in cancer cells decreases the development of bone metastases. TGF-β stimulates prostate cancer cell signaling and alters their phenotype.

TGF-β signaling in cancer is however complex and can lead to the activation of numerous genes. We identified PMEPA1 as the most highly upregulated gene by TGF-β in PC-3 cells. Although PMEPA1 has already been shown to be upregulated in different cancers, nothing is known about its function in cells. We have shown that the absence of PMEPA1 in prostate cancer cells decreases TGF-β signaling. This result is consistent with preliminary experiments showing that the cytosolic isoform of PMEPA1 which is the most highly expressed in PC-3 increases TGF-β signaling. Interestingly the other isoforms of PMEPA1 which are membrane-bound have an opposite effect, decreasing TGF-β signaling. These results suggest that depending on which isoform is the most abundant in cells, PMEPA1 can provide a positive or negative feedback loop for TGF-β signaling. We are in the process of stably knocking-down and overexpressing PMEPA1 in PC-3 cells to determine the effect of PMEPA1 on bone metastases development.

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APPENDICES

1. Abstract from the Skeletal Complications of Malignancy V Meeting.

TGF-β promotes prostate cancer bone metastases and increases expression of pro-osteolytic genes and of the TGF-β signaling regulator PMEPA1

PGJ Fournier, GA Clines, JM Chirgwin, TA Guise. Medicine/Endocrinology, University of Virginia, Charlottesville, VA, USA

Prostate cancers commonly metastasize to bone and stimulate abnormal bone resorption and bone formation. Tumors which colonize bone are exposed to high concentrations of growth factors housed in bone matrix, such as insulin-like growth factors and transforming growth factor-β **(**TGF-β**)**. These factors are released by osteoclastic resorption and fuel a vicious cycle of metastatic growth by changing the phenotype of the tumor cells. TGF-β is central to the pathogenesis of osteolytic metastases due to breast cancer and melanoma, but its role in prostate cancer bone metastases is less clear. We hypothesized that TGF-β would also promote prostate cancer bone metastases.

A specific inhibitor of the TGF-β type I receptor (TβRI) kinase, SD-208, decreased TGF-β-dependent Smad2 phosphorylation in PC-3 human prostate cancer cells *in vitro*. In vivo, PC-3 cells cause osteolytic bone metastases when inoculated into the left cardiac ventricle of male nude mice. Mice given 50mg/kg SD-208 daily had significantly decreased osteolytic bone metastases and increased survival compared to vehicle-treated mice when receiving drug from the time of tumor inoculation (prevention protocol). In a treatment protocol where mice receive drug from the time of detected osteolytic lesions, SD-208 at the same dose significantly decreased malignant osteolysis but did not improve survival. Therefore,TGF-β also promotes prostate cancer bone metastases.

To determine genes regulated by TGF-β, we analyzed PC-3 cells treated with TGF-β (24h, 5ng/mL) by Affymetrix gene array using DMT and dCHIP data analyses. Significantly upregulated genes included known TGF-β targets PTHrP, CTGF, MMP-13, TSP-1 and ADAM19, which function in bone remodeling or are dysregulated in cancer. The most increased gene was PMEPA1 (23.2-fold, $P<0.03$), a protein highly expressed in breast, colon and prostate cancers. Using real-time RT-PCR of RNA from PC-3 cells treated with TGF-β (5ng/mL, for 0 to 48h), we confirmed that PMEPA1 mRNA was rapidly induced and peaked at 24h (16.7-fold, $P<0.05$). TGF-β also increased PMEPA1 mRNA in prostate, breast and lung cancer lines. Treatment of PC-3 cells with SD-208, actinomycin D, or cycloheximide showed that TGF-β directly activates PMEPA1 transcription. TGF-β also increased PMEPA1 protein in PC-3 by Western blot; the induction was prevented by SD-208. We cloned and made deletion mutants in 3.7kb of the human PMEPA1 promoter, which contains 5 putative Smad-binding elements. Dual-luciferase assays and overexpression of Smads 2, 3 and 4 or inhibitory Smad7, indicated that PMEPA1 transcription is regulated by TGF-β via both Smad-dependent and -independent pathways.

PMEPA1 binds to the E3 ubiquitin ligase Nedd4, a relative of the Smurf proteins that inhibit TGF-β signaling, suggesting that PMEPA1 could regulate TGF-β signaling. Multiple PMEPA1 transcripts encode 3 protein isoforms with differing N-termini: 2 with a transmembrane domain, while the 3rd one is cytosolic. Sequences encoding each of the isoforms were expressed in A549 lung cancer cells to test their effects on the Smad-responsive $(CAGA)_9$ promoter. The membrane-bound PMEPA1 isoforms significantly inhibited the TGF-β-induced luciferase activity, while the cytosolic isoform did not. The membrane bound isoforms also reduced the Smad7-mediated inhibition of $(CAGA)_9$ promoter activity. The results suggest that membrane localization is required for the PMEPA1 inhibition of Smad-mediated TGF-β signaling.

Preliminary data suggest that cytosolic PMEPA1 is the most abundant TGF-β-induced isoform in PC-3 prostate cancer cells. We hypothesize that bone-derived TGF-β acts on metastatic prostate cancer cells to increase the non-inhibitory, cytosol isoform of PMEPA1, thereby potentiating TGF-β signaling and enhancing bone metastases.

2. Abstract from the 25th Annual American Cancer Society Virginia Cancer Researchers Seminar.

TGF-β promotes prostate cancer bone metastases and increases the expression of pro-osteolytic genes and of the TGF-β signaling regulator PMEPA1

PGJ Fournier, GA Clines, JM Chirgwin, TA Guise. Medicine/Endocrinology, University of Virginia, Charlottesville, VA, USA.

Prostate cancers commonly metastasize to bone stimulating abnormal bone resorption and bone formation. Tumors which colonize bone are exposed to high concentrations of growth factors. These are released during the osteoclastic resorption and fuel a vicious cycle of metastatic growth. Transforming growth factor-β (TGF-β) has been implicated as central in bone metastases from breast cancer and melanoma, but its role in prostate cancer bone metastases has been less studied.

We used an inhibitor specific of the TGF-β type I receptor (TβRI) kinase domain, SD-208, to test the role of TGF-β in prostate cancer bone metastases. SD-208 inhibited Smad2 phosphorylation induced by TGF-β in PC-3 prostate cancer cells *in vitro*. In a mouse bone metastases model, human PC-3 prostate cancer cells cause osteolytic bone metastases when inoculated into the left cardiac ventricle of male *nude* mice. Mice treated with SD-208, 50mg/kg/day, had significantly less osteolysis due to bone metastases and increased survival compared with vehicle-treated mice. This was evident when mice received drug at either the time of tumor inoculation (prevention setting) or at the time of established osteolytic lesions (treatment setting).

To determine downstream targets of TGF-β in prostate cancer, we analyzed PC-3 cells treated with TGF-β (24h, 5ng/mL) by Affymetrix gene array. Significantly upregulated genes included the known TGF-β targets PTHrP, CTGF, MMP-13, TSP-1 and ADAM19, which function in bone remodeling or are dysregulated in cancer.

The most increased gene was PMEPA1 (23.2-fold), a protein highly expressed in breast, colon and ovarian cancers. We validated that PMEPA1 mRNA was rapidly induced by TGF-β and peaked at 4 hours. TGF-β also increased PMEPA1 protein production in PC-3. PMEPA1 is expressed and up-regulated in other prostate, breast and lung cancer cells. Treating PC-3 cells with the classical actinomycin D or cycloheximide showed that TGF-β directly activates PMEPA1 transcription. Dual-Luciferase analysis of a 3.7kb fragment of PMEPA1 promoter and deletion fragments indicated that TGF-β regulates PMEPA1 transcription via Smad and non-Smad mechanisms. PMEPA1 interacts with the E3 ubiquitin ligase Nedd4 related to the Smurfs which inhibit TGF-β signaling by targeting Smads and TβRI for proteasomal degradation. A sequence homolog to the Smad Interaction Motif of DNA-binding cofactors was also identified in the C-terminus of PMEPA1 suggesting direct interaction with Smad proteins. PMEPA1 could then regulate TGF-β signaling. Using shRNAs we observed that PMEPA1 knock-down in PC-3 cells decreased TGF-β signaling.

We hypothesize that at sites of bone metastases PMEPA1 induced by bone-derived TGF-β will potentiate TGF-β signaling in cancer cells and further enhance bone metastases.

3. Abstract from the FASEB Summer Research Conferences. TGF-β Superfamily: Signaling and Development.

TGF-β promotes prostate cancer bone metastases and increases expression of pro-osteolytic genes and of the TGF-β signaling regulator PMEPA1

PGJ Fournier, GA Clines, JM Chirgwin, TA Guise. Medicine/Endocrinology, University of Virginia, Charlottesville, VA, USA.

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4. Abstract from the American Society for Bone and Mineral Research 29th annual meeting.

TGF-β increases osteolytic prostate cancer bone metastases and expression of pro-metastatic genes

PGJ Fournier, GA Clines, JM Chirgwin, TA Guise
Endocrinology, University of Virginia, Charlottesville, VA, USA

Prostate cancers commonly metastasize to bone, where high concentrations of TGF-β are released by osteoclastic resorption. TGF-β stimulates production of PTHrP, IL-11 and CTGF, which are central factors in bone metastases due to breast cancer and melanoma. We hypothesized that TGF-β would also promote prostate cancer bone metastases.

First, we showed that a specific inhibitor of the TGF-β type I receptor (TβRI) kinase, SD-208, inhibited TGF-β-dependent Smad2 phosphorylation in PC-3 prostate cancer cells *in vitro*. Mice were inoculated with PC-3 cells via the left cardiac ventricle. In both prevention and treatment protocols, SD-208 (50mg/kg/d) decreased osteolytic metastases and increased survival. To determine downstream targets of TGF-β in prostate cancer, we analyzed PC-3 cells treated with TGF-β (24h, 5ng/mL) by Affymetrix gene array with

DMT and dCHIP data analyses. Significantly upregulated genes included known TGF-β targets PTHrP, CTGF, MMP-13, TSP-1 and ADAM19, which function in bone remodeling or are dysregulated in cancer.

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PMEPA1 interacts with the E3 ubiquitin ligase Nedd4, which is related to the Smurfs. These proteins inhibit TGF-β signaling by targeting Smads and TβRI for proteasomal degradation. PMEPA1 could prevent proteasomal inhibition of the TGF-β pathway by suppressing Nedd4/Smurf activity, leading to sustained TGF-β signaling in bone metastases. Our data indicate that TGF-β promotes osteolytic bone metastases by stimulating known prometastatic factors, as well as novel factors that may enhance TGF-β signaling in the tumor cell. Thus, TGF-β inhibitors should be effective treatments for osteolytic prostate cancer bone metastases.

SUPPORTING DATA

cf pages 17 to 31

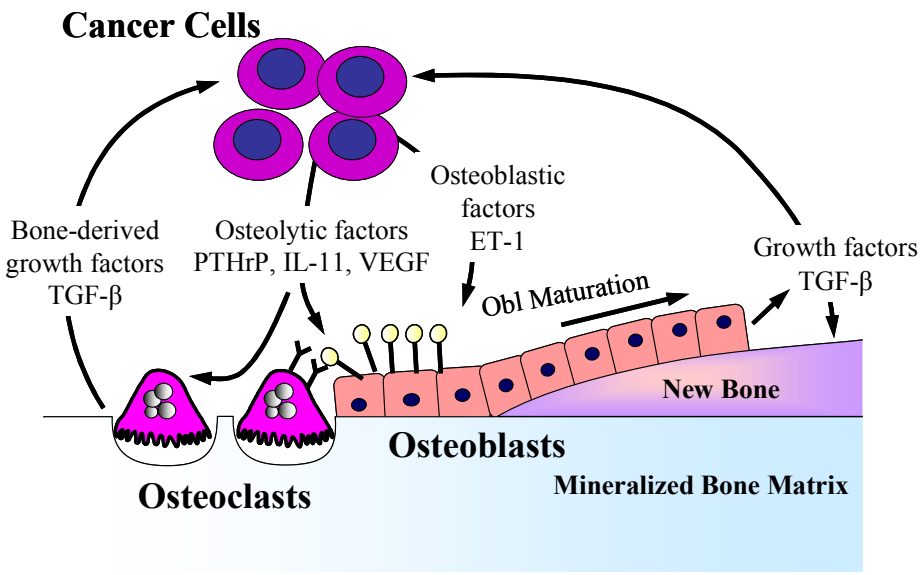


Figure 1. Tumor-bone cell interactions in the vicious cycle of bone metastases. Tumor stimulation of osteoblasts can increase both new bone formation and resorption. Tumor products, such as ET-1, stimulate osteoblasts proliferation. Immature osteoblasts respond to osteolytic cytokines, such as PTHrP, by expressing RANK ligand. RANK ligand stimulates bone resorption by osteoclasts, which release growth factors from mineralized matrix. Mature osteoblasts synthesize growth factors stimulate tumor cells. TGF-β is one of the major factors synthesized by osteoblasts, stored in mineralized bone matrix, and released by osteoclastic bone resorption.

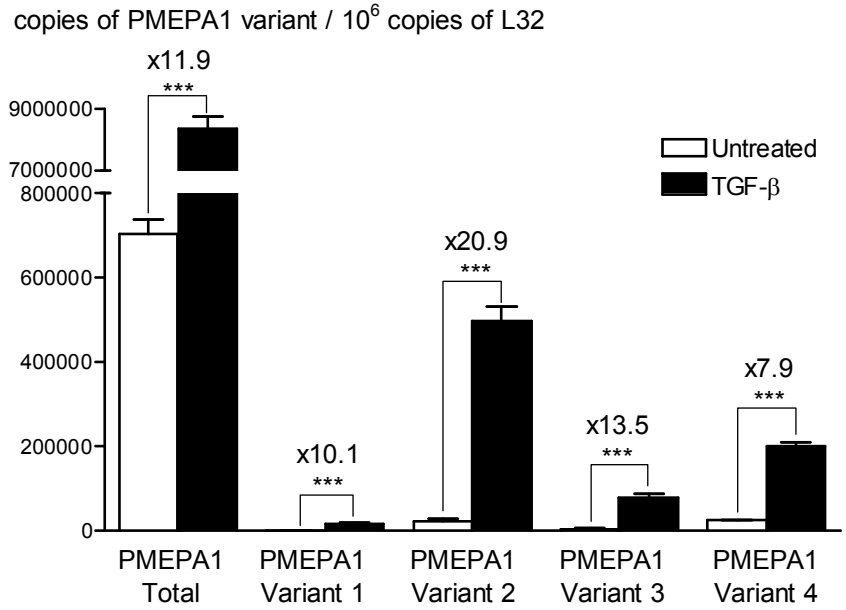


Figure 2. TGF-β increases the expression of all PMEPA1 variants in PC-3 prostate cancer cells. PC-3 cells were treated or not treated with TGF-β (5ng/mL, 24h) before extracting total RNA and preparing cDNA. Expression of each variant of PMEPA1 was quantified using real-time PCR and normalized to the endogenous ribosomal protein L32. Results are shown as the average ± SD of 3 independent experiments. ***, *P*<0.001 when compared to the untreated cells, using a non-parametric Mann-Whitney's U test.

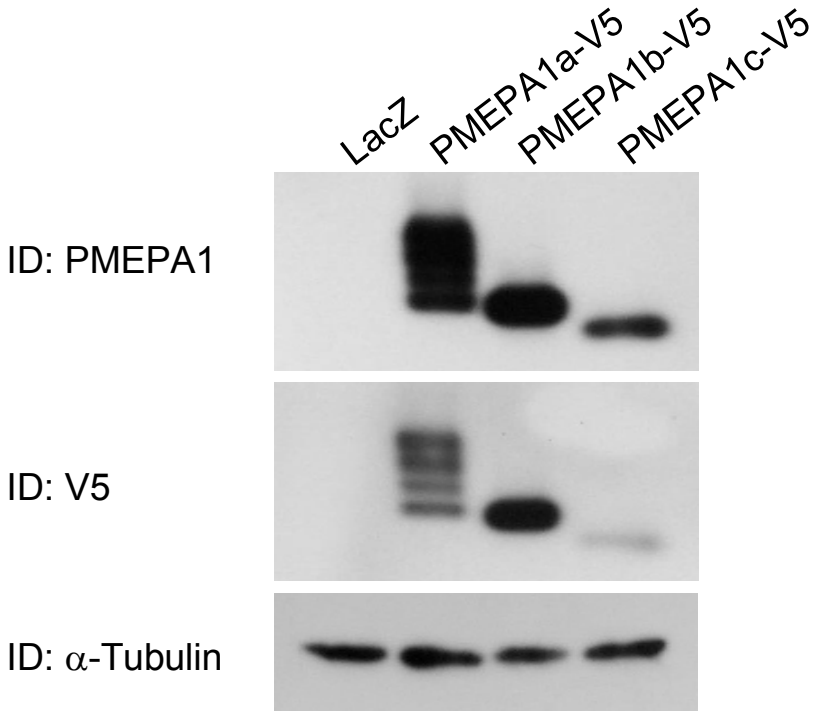


Figure 3. Validation of the antibody against PMEPA1. COS-1 were transfected with plasmid coding for LacZ, or the different isoforms of the PMEPA1 proteins tagged with V5. Protein lysates were analyzed by Western-blotting against PMEPA1. After stripping, the membrane was probed for the epitope V5 and for α-tubuline.

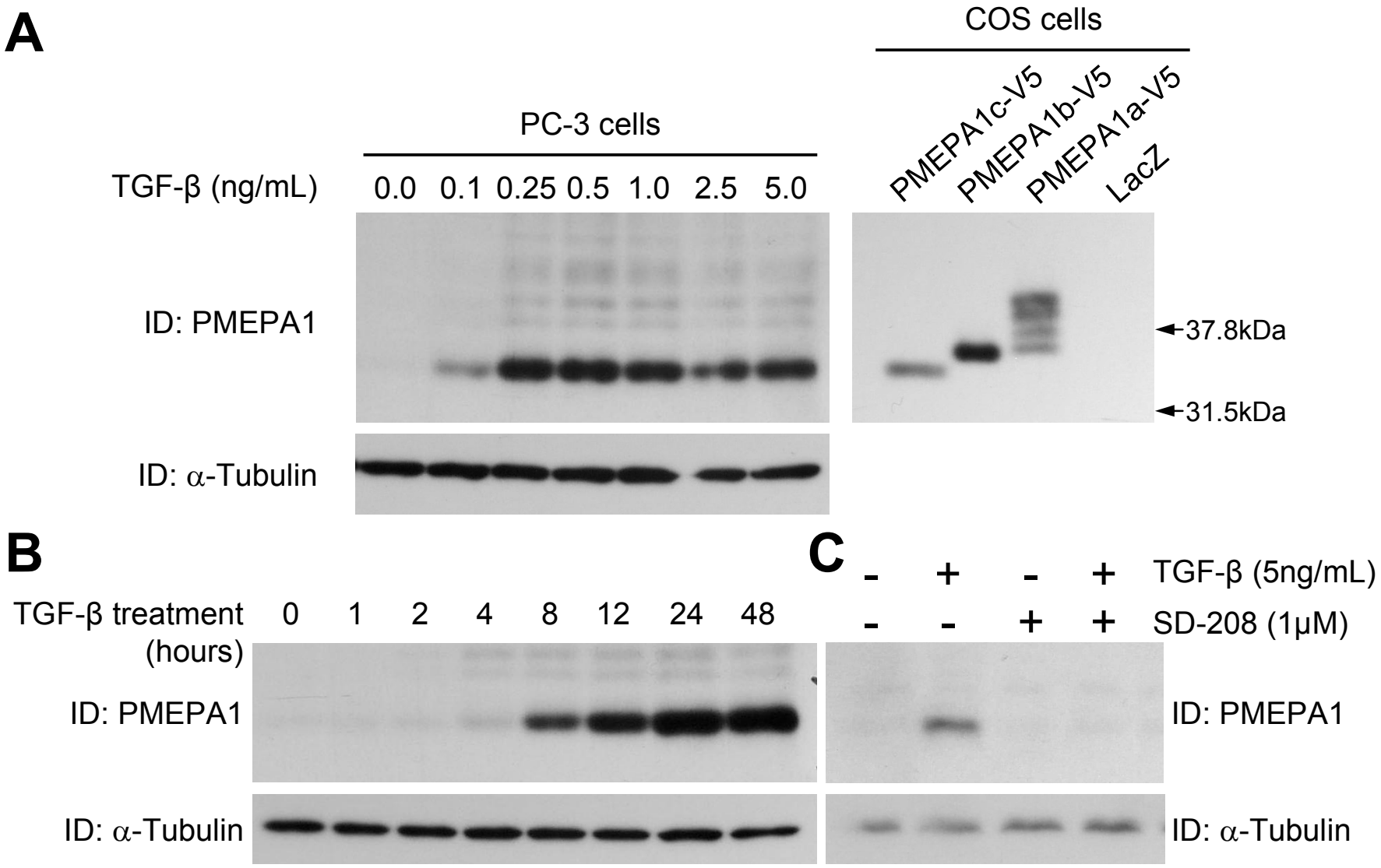


Figure 4. TGF-β induces a dose- and time-response increase of PMEPA1c protein in PC-3 cells. A. PC-3 cells were treated or not treated with 0.1 to 5.0ng/mL TGF-β for 24 hours before preparing protein lysates analyzed by Western-blotting, next to protein lysates of COS cells transfected to express LacZ or one of the PMEPA1 isoform. **B.** PC-3 cells were treated or not treated with 5ng/mL TGF-β from 1 to 48 hours before preparing protein lysates. **C.** PC-3 cells were pre-treated or not with the Tgfbr1 inhibitor SD-208(1μM) before being treated or not treated with TGF-β for 24 hours. Protein lysate were analyzed by Western-Blot to immunodetect PMEPA1. After stripping, the membrane was probed for α-Tubulin.

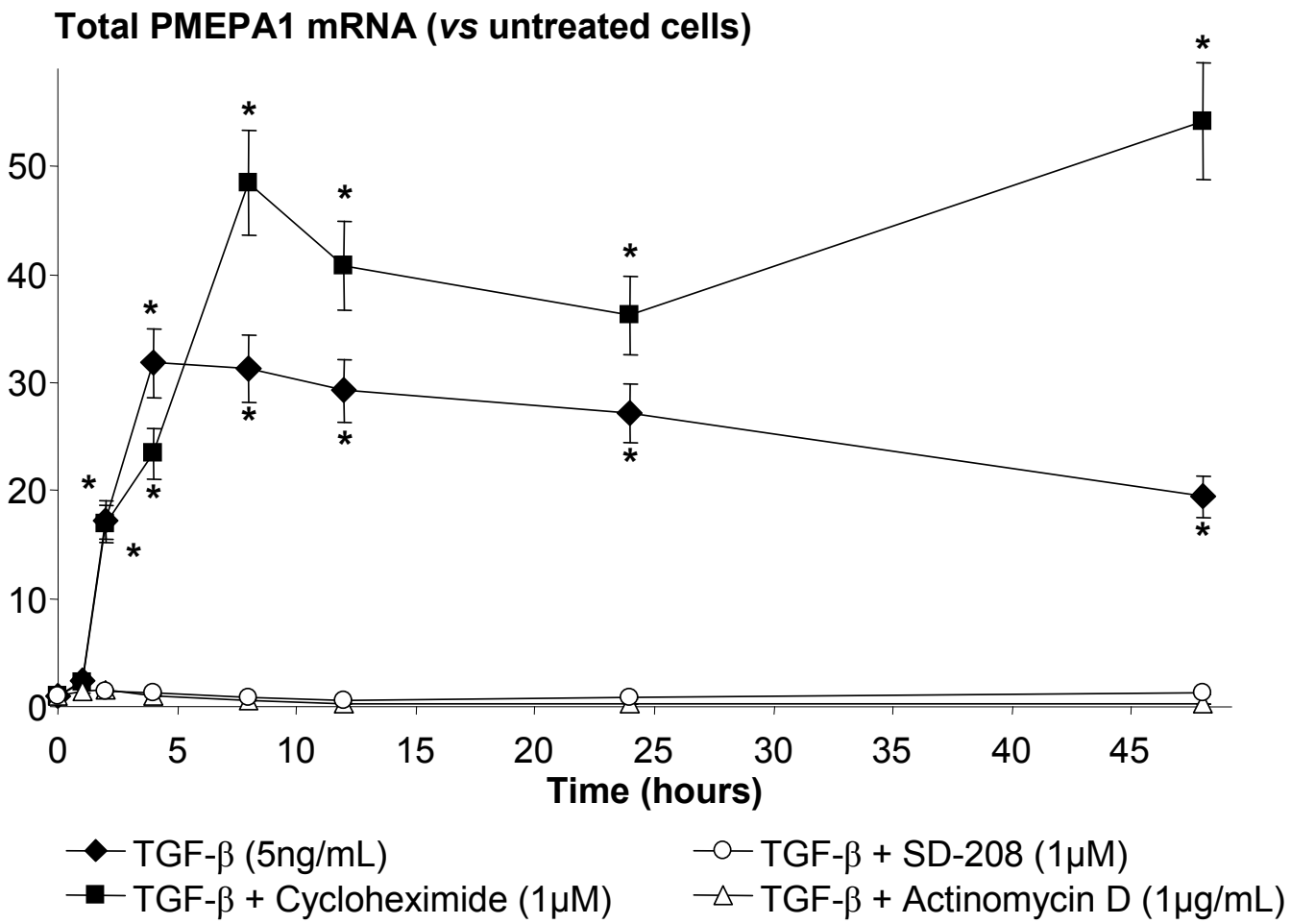
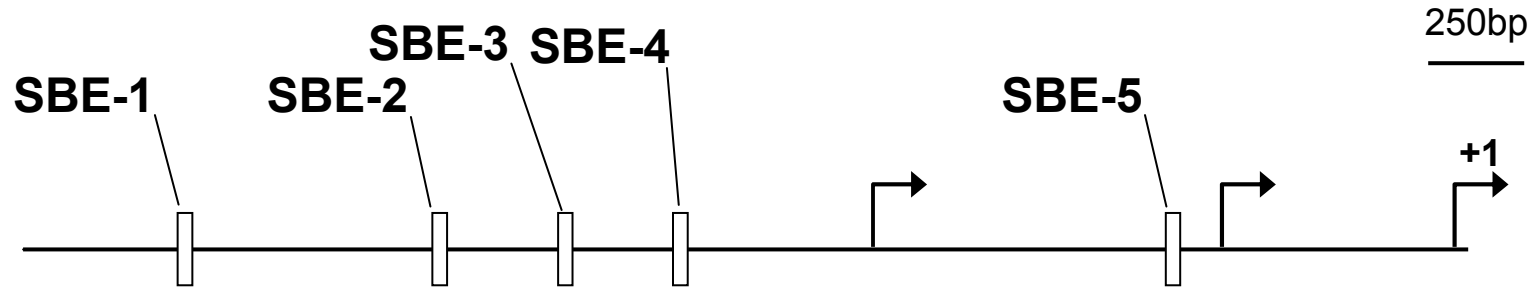


Figure 5. TGF-β increases PMEPA1 gene transcription. PC-3 cells were cultured until near confluency and starved overnight. Cell culture medium was then substituted with RPMI 1640-FBS (1%) and cells were treated or not treated with TGF-β for 0 to 48 hours, in the presence or absence of cycloheximide (translation inhibitor), actynomicin-D (transcription inhibitor) or SD-208 (Tgfbr1 inhibitor). Total RNA were extracted and cDNA were prepared. Total PMEPA1 expression was quantified by real-time PCR and normalized using the endogenous ribosomal protein L32 transcript, using the ΔΔCt method. All conditions were run in triplicate. The results represent the average (±SD) increase of PMEPA1 transcript induced by TGF-β and were analyzed using an unpaired Student's *t* test (*, $P<0.01$ when compared to untreated cells).



↱ Transcription start

▯ 5'-CAGACA-3' Smad binding element (SBE)

Figure 6. Schematization of a 3.7kb fragment of the PMEPA1 promoter.

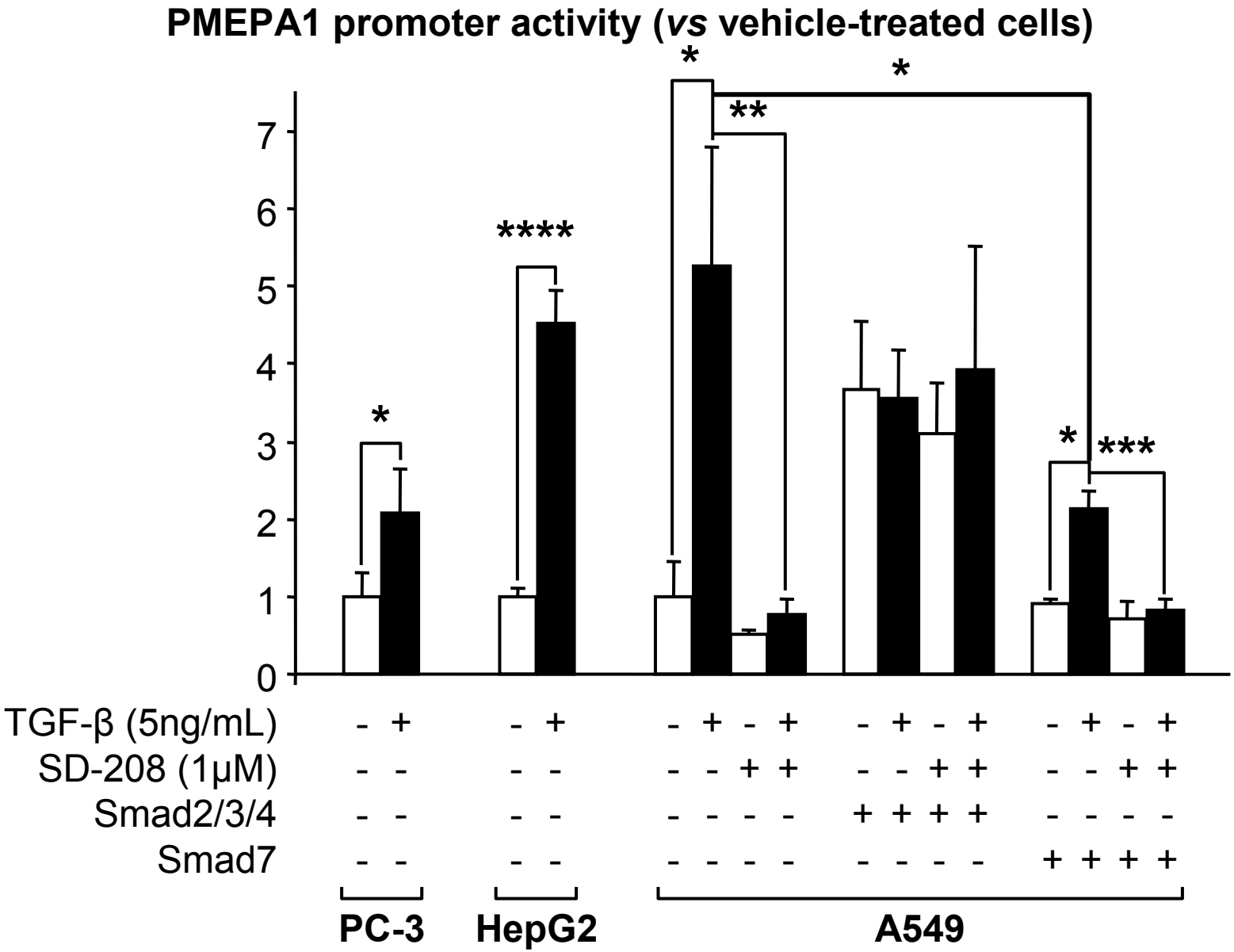


Figure 7. TGF-β-induction of PMEPA1 promotor activity is regulated by Smad proteins. ph*r*Luc-CMV plasmid and a pGL3-*f*Luc reporter plasmid containing the 3.7kb fragment of PMEPA1 promoter were transfected in PC-3, HepG2 or A549 cells with or without pCMV5 plasmids expressing Smad2/3/4 or Smad7. Cells were treated ± TGF-β and SD-208 for 24 hours before measuring dual-luciferase activity. Average ± SD of one representative experiment performed in quadruplicate. **P*<0.05, ***P*<0.01, ****P*<0.005, *****P*<0.001, using an unpaired, two-tailed Student's *t* test.

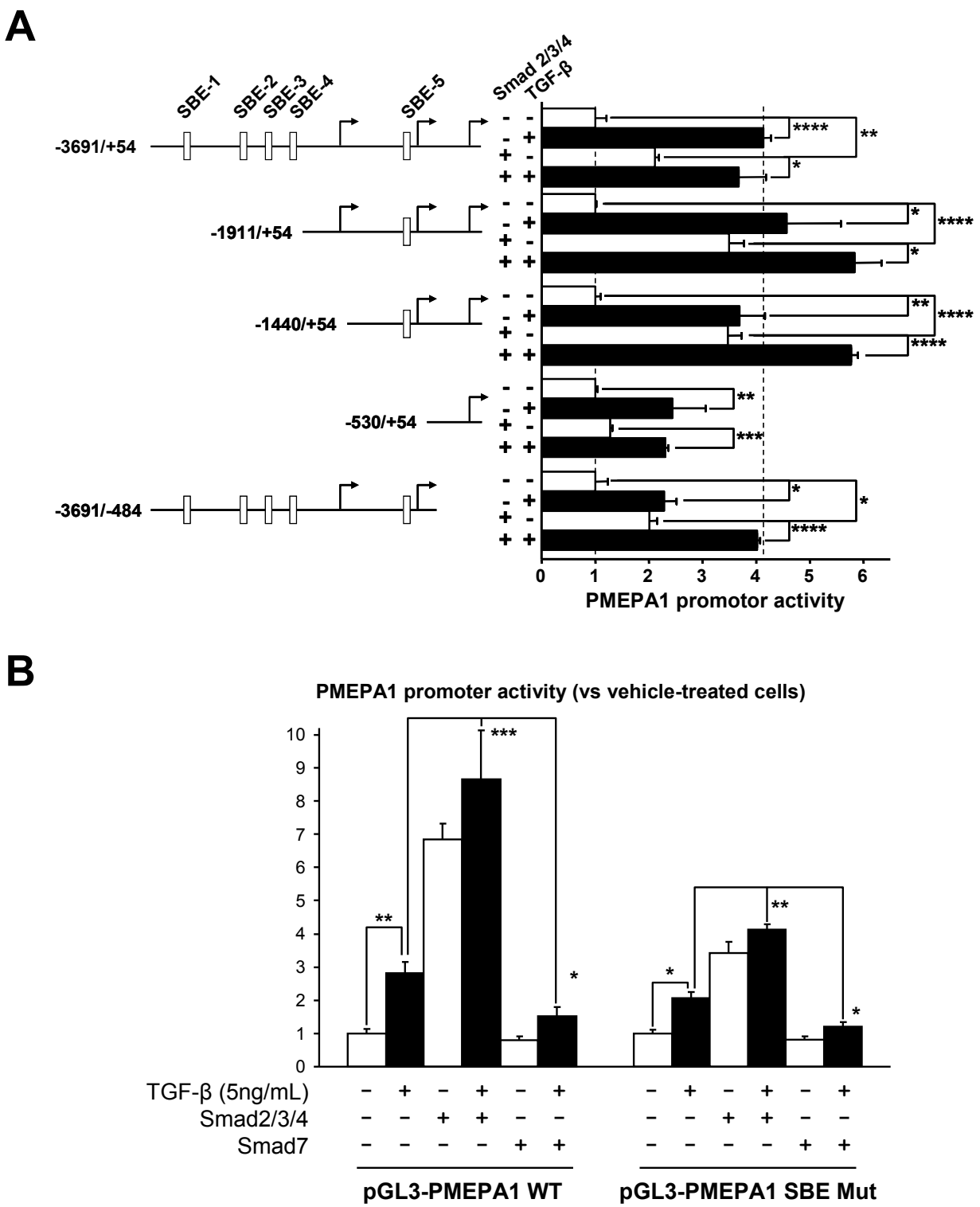


Figure 8. Mutation of the SBE in the PMEPA1 promoter does not decrease TGF-β responsiveness. A. ph*r*Luc-CMV plasmid and a pGL3-*f*Luc reporter plasmid containing the PMEPA1 promoter with or without mutated SBE (pictured by the crossed boxes) were transfected in A549 cells. **B.** A549 cells were transfected with phrLuc-CMV and a pGL3-PMEPA1 wild-type (WT) or where the five SBEs have been mutated (SBE Mut). Cells were treated ± TGF-β for 24 hours before measuring dual-luciferase activity. **P*<0.05, ***P*<0.01, ****P*<0.005, *****P*<0.001, using an unpaired, two-tailed Student's *t* test.

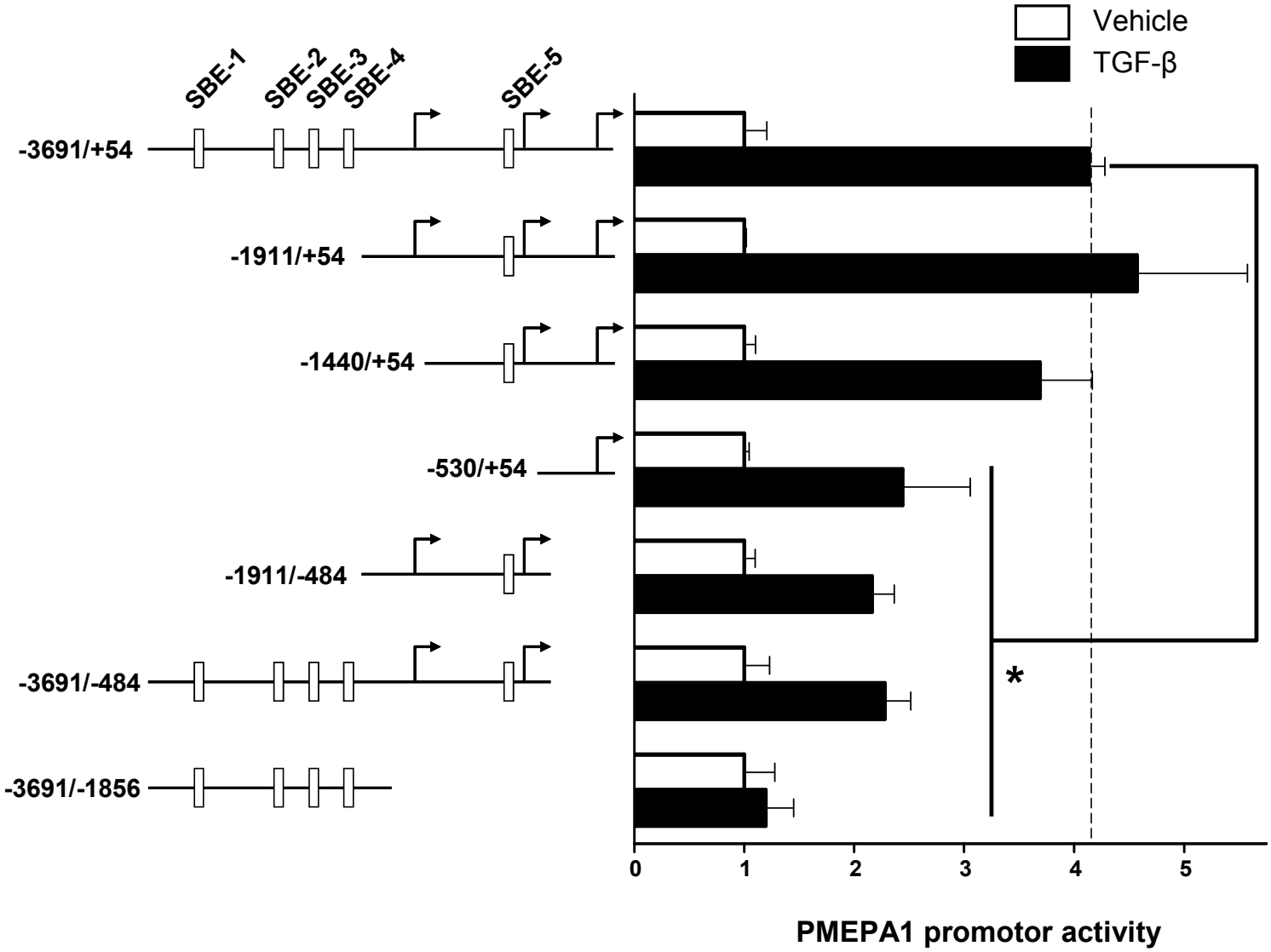


Figure 9. TGF-β-induction of PMEPA1 promoter is mediated within a 1.5kb proximal fragment of the promoter. A549 cells were transfected with a pGL3-fLuc reporter plasmid containing a fragment of the PMEPA1 promoter and a phrLuc-CMV plasmid, and treated ± TGF-β (5ng/mL, 24h) before measuring dual-luciferase activity. Average ± SD of one representative experiment performed in quadruplicate. * $P<0.05$, using a non-parametric Mann Whitney's *U* test.

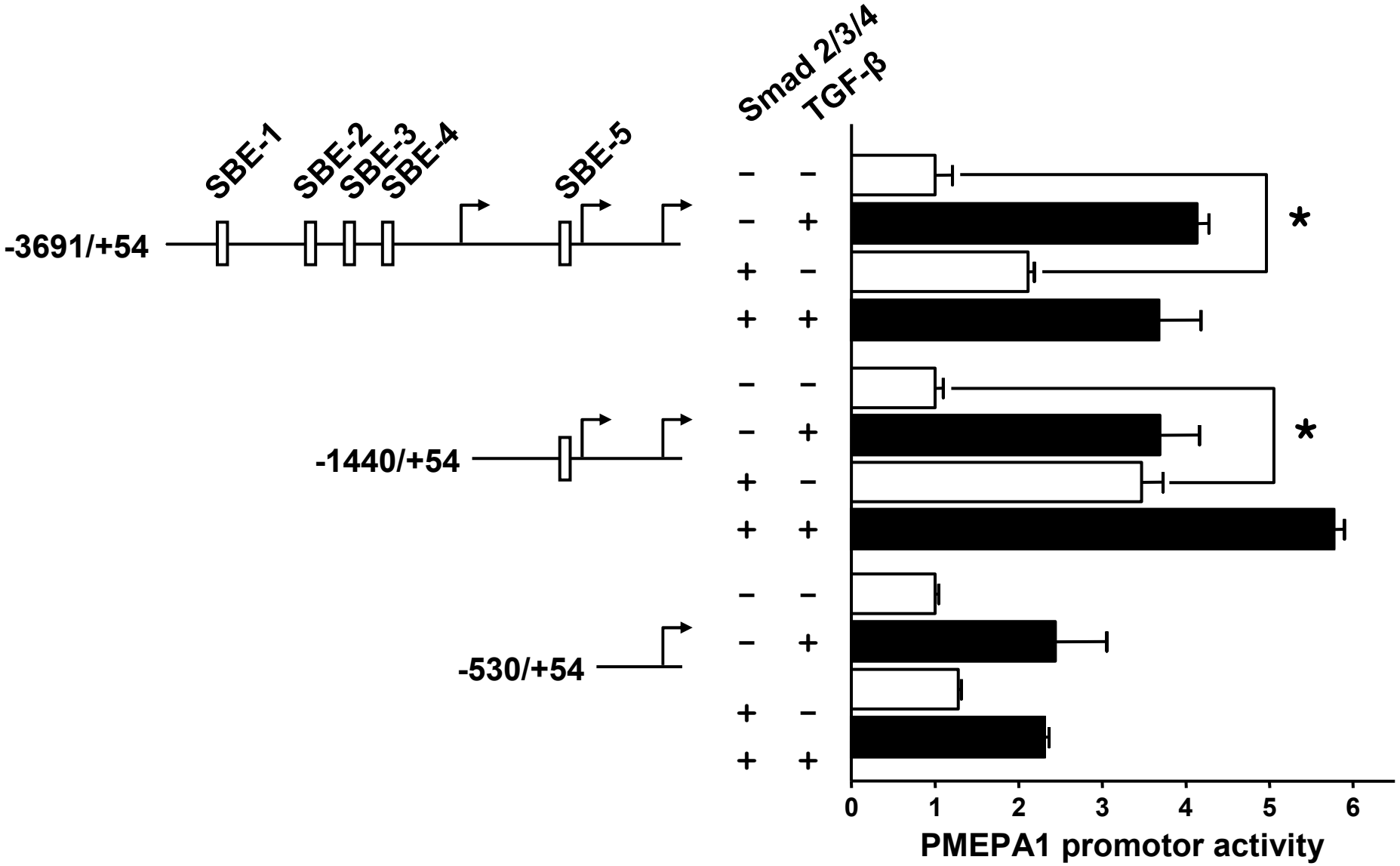


Figure 10. TGF-β-induction of PMEPA1 promoter is Smad and non-Smad regulated. A549 cells wee transfected with a pGL3-fLuc reporter plasmid containing a fragment of the PMEPA1 promoter, a phrLuc-CMV plasmid and pCMV vectors expressing Smad2, 3 and 4 or an empty pCMV vector. Cells were treated ± TGF-β (5ng/mL, 24h) before measuring the dual-luciferase activity. Average ± SD of one representative experiment performed in quadruplicate. * $P<0.05$, using a non-parametric Mann Whitney's *U* test.

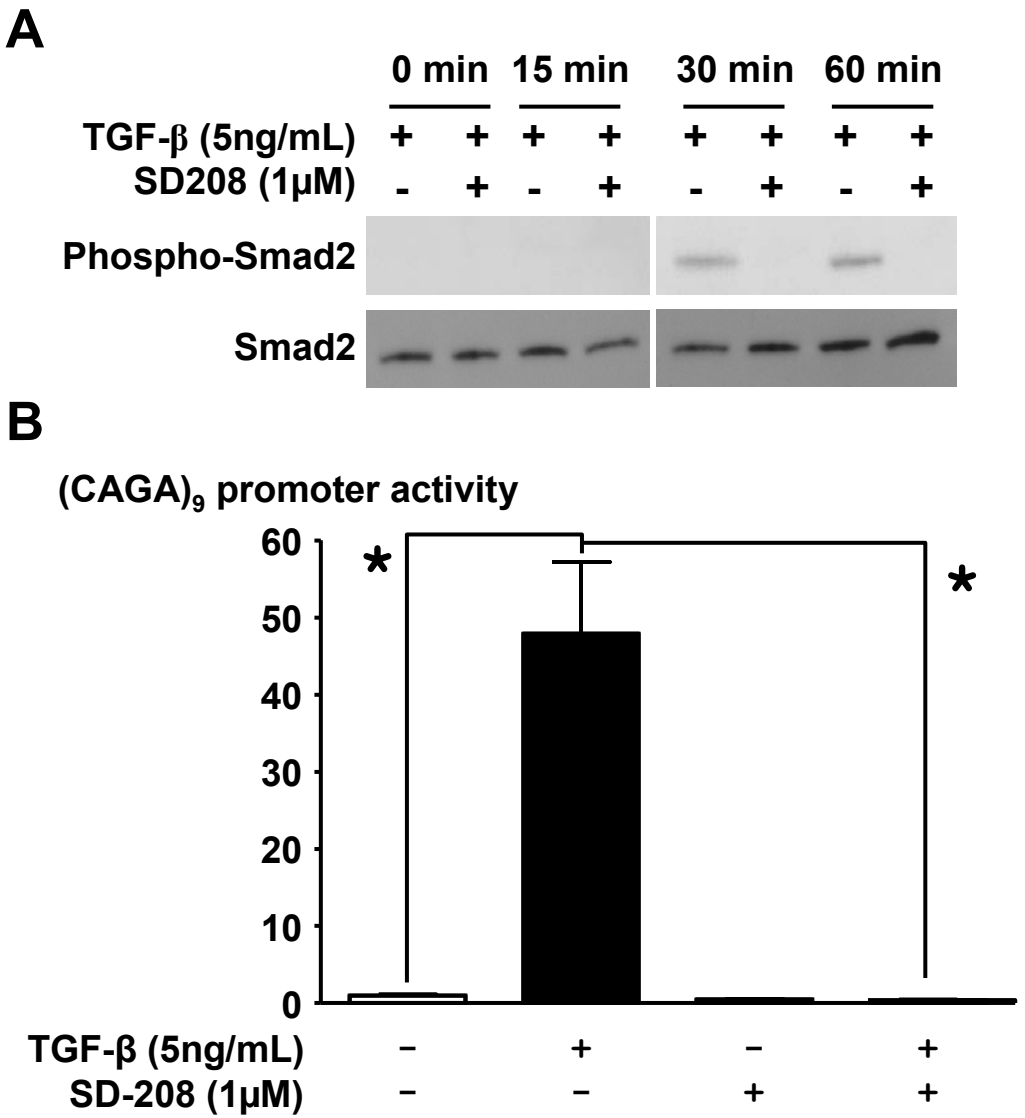


Figure 11. SD-208 inhibits TGF-β signaling in PC-3 cells. A. Immunoblotting detection of Smad2 and phospho-Smad2 on protein lysate from PC-3 cells treated or not with TGF-β, in the presence or absence of SD208. **B**. PC-3 cells were transfected with the pGL3 (CAGA)9-*f*Luc reporter construct sensitive to TGF-β and a *r*Luc vector. Cells were treated ± TGF-β (5ng/mL) for 24 hours before measuring dual-luciferase activity. *P<0.05 using a non-parametric Mann-Whitney's U test.

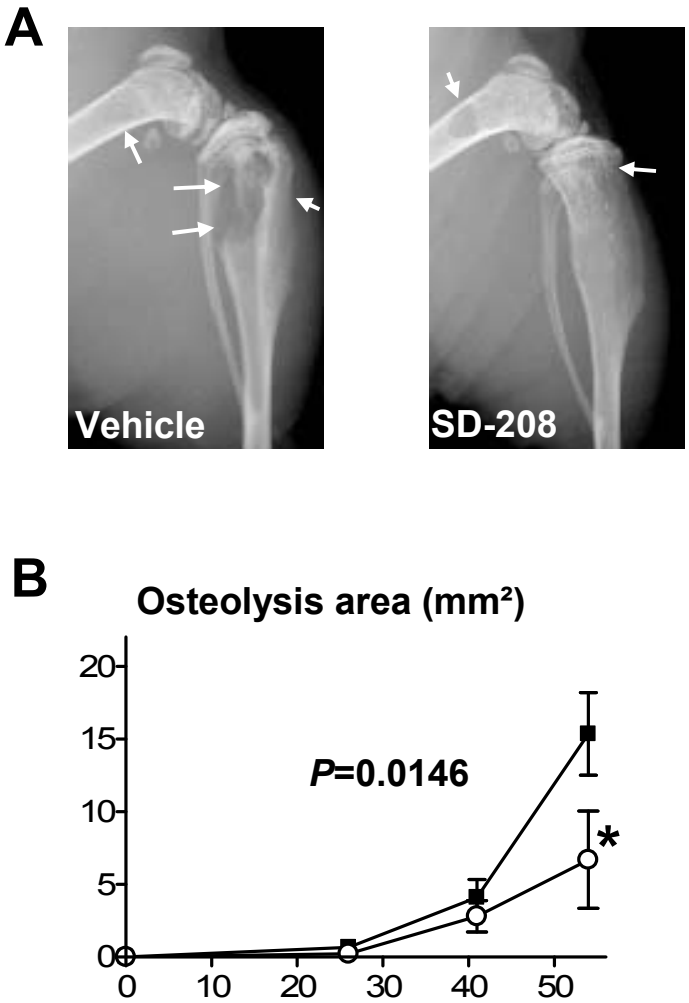


Figure 12. Inhibition of TGF-β signaling decrease bone destruction induced by PC-3 cells. A. Representative radiographies of tibia and distal femur of mice treated with vehicle or SD-208. Arrows indicate osteolytic area. **B.** Quantification of bone destruction. Results are expressed as the average osteolysis area ± SEM, and statistically analyzed using a 2-way ANOVA test.

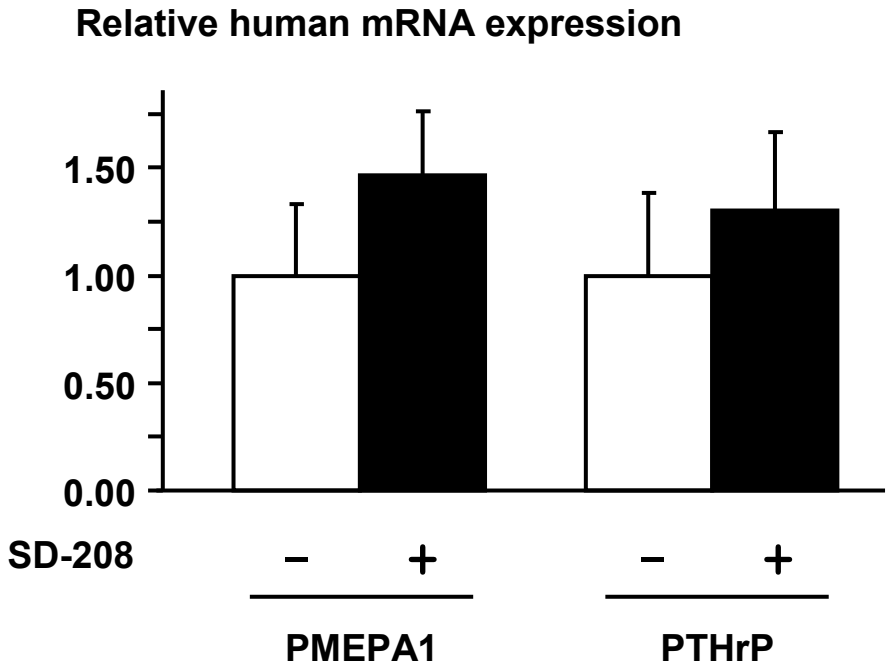


Figure 13. SD-208 (50mg/kg/day) does decrease human PMEPA1 or PTHrP mRNA at sites of bone metastases. Human gene expression was measured in bone marrow mRNA samples from mice with bone metastases from PC-3 cells treated or not treated with SD-208 (50mg/kg/day).

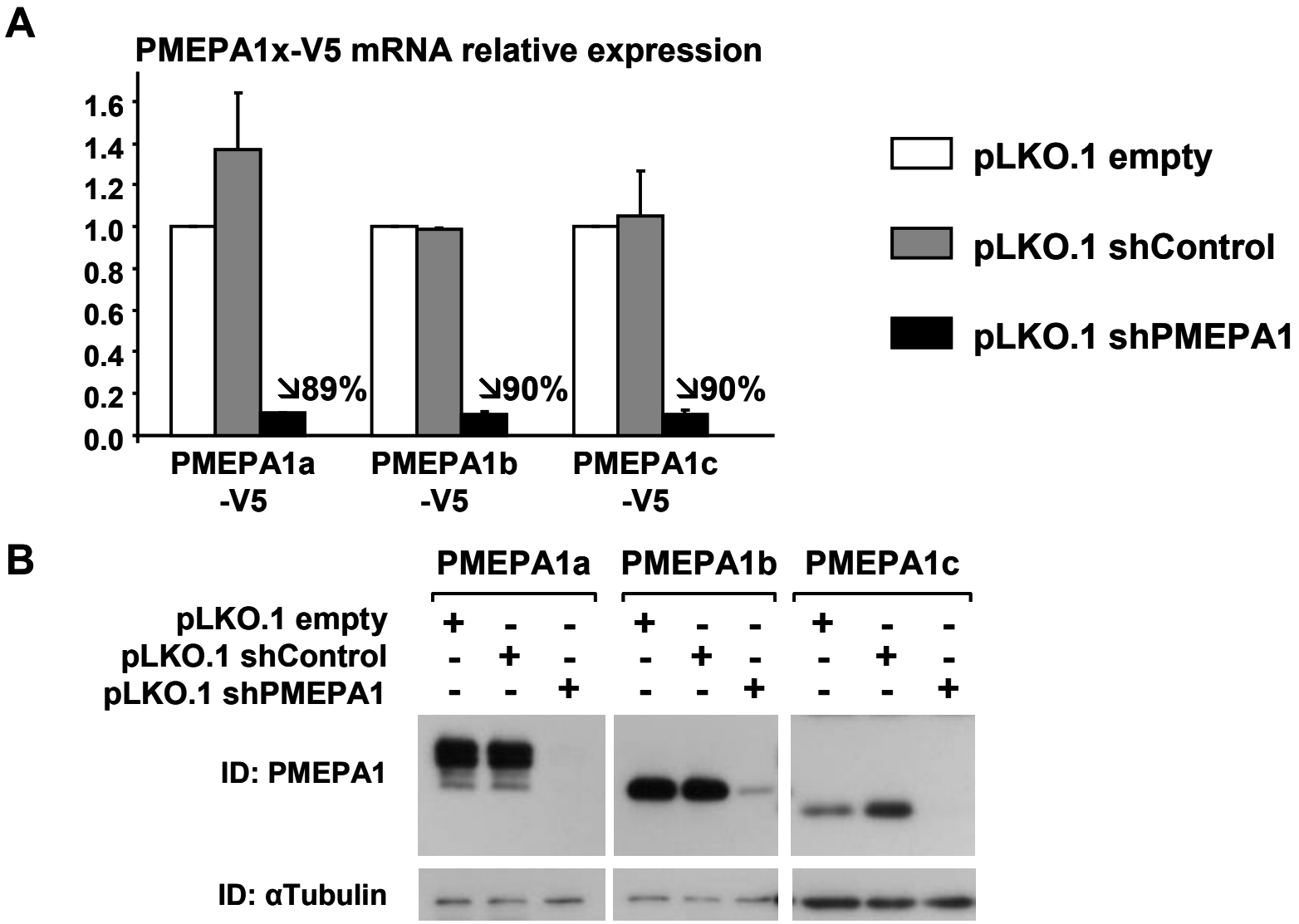


Figure 14. Validation of a short-hairpin RNA against PMEPA1. COS7 cells were trasnfected with a pcDNA vector expressing different isoforms of PMEPA1 with a V5 tag at the C-terminus and with an empty pLKO.1 vector or expressing a shRNA control (shControl) or a shRNA against PMEPA1 (shPMEPA1). Forty-eight hours later, (**A**) total RNA was extracted to measure PMEPA1 mRNA levels using sqRT-PCR and (**B**) protein lysate were prepared to determine PMEPA1 protein levels using Western-blotting to detect PMEPA1 and αTubulin.

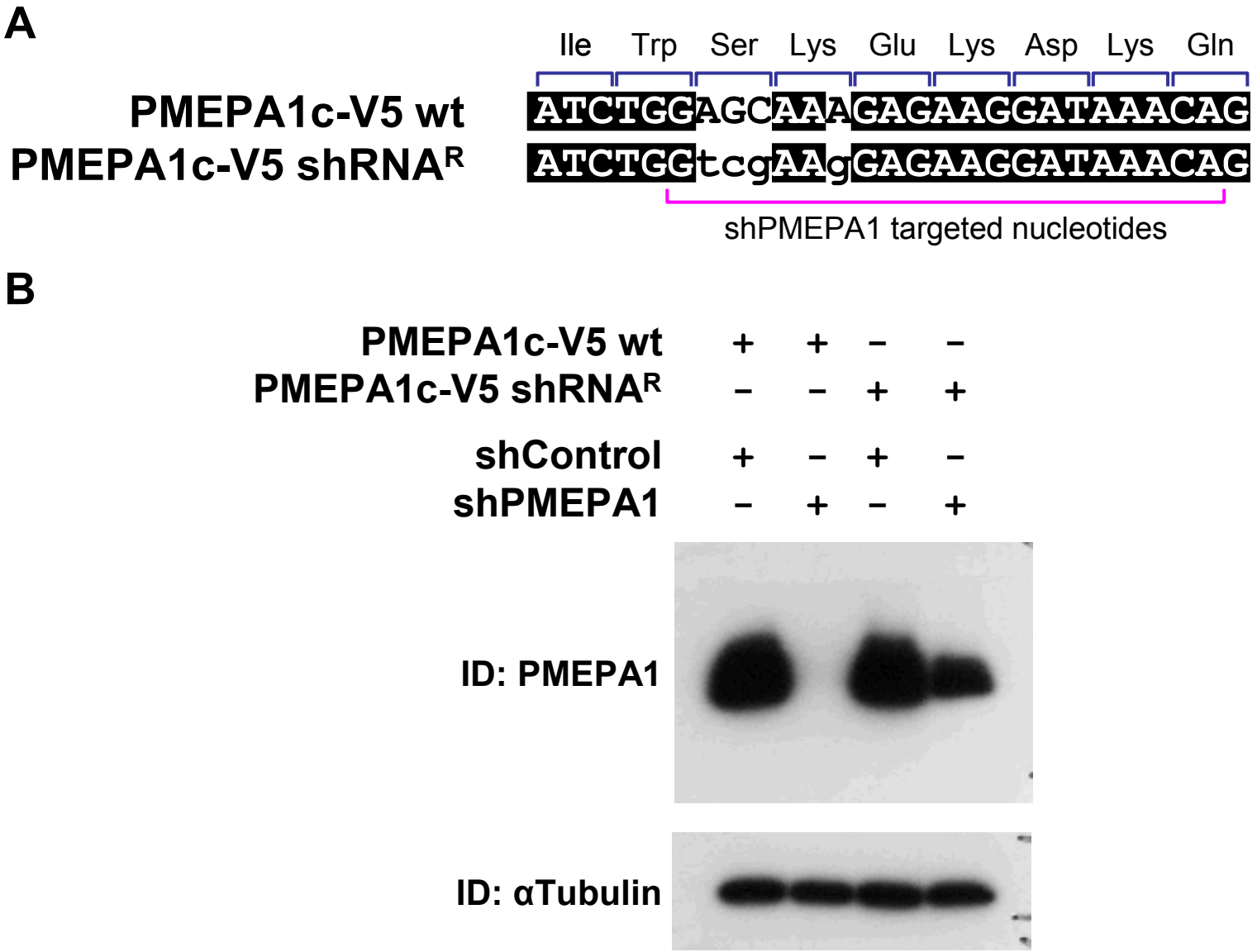


Figure 15. Generation of a shRNA-resistant form of the PMEPA1c isoform. A. Silent mutations introduced in the coding sequence of PMEPA1c at the site recognized by the shPMEPA1. **B**. pcDNA plasmid coding for the wild-type (wt) or the shRNA resistant (shRNA^R) PMEPA1c-V5 were cotransfected in COS7 cells with a pLKO.1 vector expressing a shControl or a shPMEPA1. Forty-eight hours later, protein lysates were prepared and analyzed using Western-blotting to detect PMEPA1 and αTubulin.

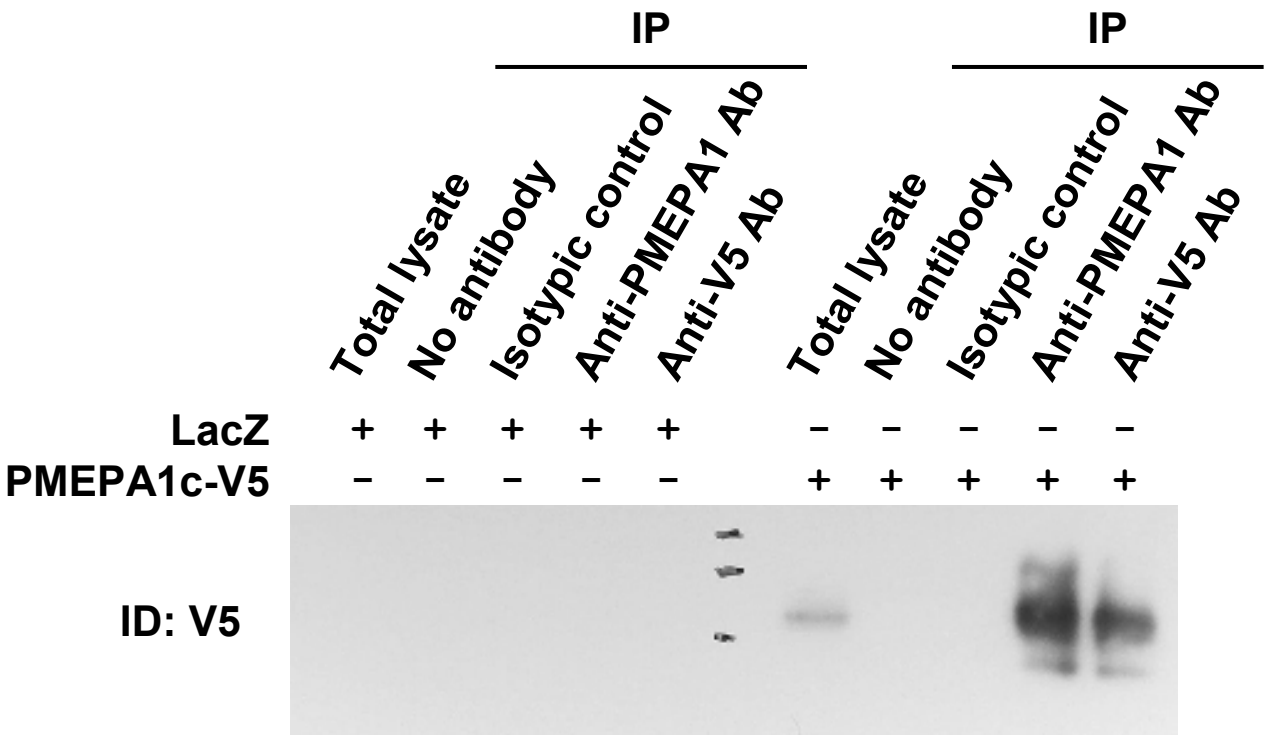


Figure 16. Validation of the mouse monoclonal antibody against PMEPA1 for immunoprecipitation. COS7 cells were transfected with a pcDNA plasmid expressing LacZ or PMEPA1c-V5. Forty-eight hours later, cells were lyzed and immunoprecipitation (IP) was performed using protein G-Sepharose in the absence of antibody or using an antibody against PMEPA1, against the epitope tag V5 or an isotypic control. Total lysate and immunoprecipitate were analyzed using Western-blotting to detect V5. PMEPA1 was detected only on total lysate or in immunoprecipitate where the anti-PMEPA1 antibody was used. No signal was detected in the absence of PMEPA1 expression (LacZ transfected cells).

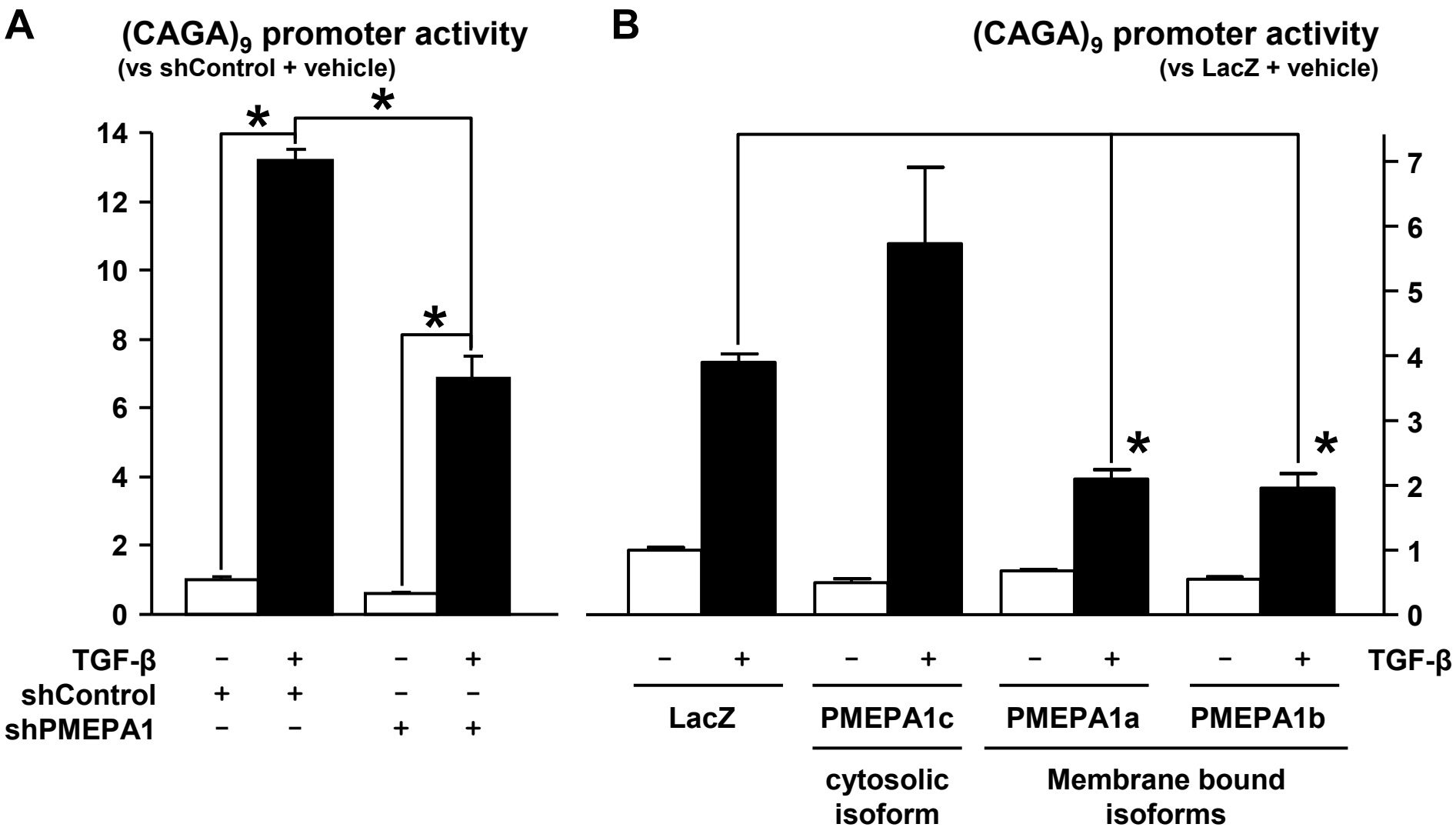


Figure 17. Effect of PMEPA1 knockdown and overexpression on TGF-β signaling. A. PC-3 cells were cotransfected with a pGL3-MLP-$(CAGA)_9$-fLuc vector sensitive to TGF-β, a phRL-CMV plasmid and a pLKO.1 vector expressing shControl or shPMEPA1. Cells were treated ± TGF-β (5ng/mL, 24h) before measuring dual-luciferase activity. **B**. PC-3 cells were cotransfected with a pGL3-MLP-$(CAGA)_9$-fLuc vector sensitive to TGF-β, a phRL-CMV plasmid and a pcDNA plasmid expressing LacZ or the isoform a, b or c of PMEPA1. Cells were treated ± TGF-β (5ng/mL, 24h) before measuring dual-luciferase activity. Average ± SD of one representative experiment performed in quadruplicate. *P<0.05 using a non-parametric Mann-Whitney's U test.